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Immune monitoring of Vaccinated Patient BV01 with Metastatic Breast Cancer
Department of Defense Grant # DAMD17-03-1-0487
Annual Progress Report
July 2011
[for the period of 07/01/2010 – 06/30/2011]

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Introduction

The main objective of the study is to vaccinate patients with metastatic breast cancer with a viable dendritic cell (DC)/breast cancer fusions in conjunction with IL-12 to induce an immunological response with the hope that this combination would further enhance vaccine response by promoting Th1 cytokine induction and T cell activation. In this approach, the entire repertoire of tumor antigens, including those yet to be identified, are expressed with the immune-stimulating machinery of the DCs. The fusion cell vaccine allows for induction of helper T and CTL responses by class II presentation of exogenous protein and class I presentation of newly synthesized endogenous protein. Several of our clinical studies have demonstrated that vaccination with fusion cells was well tolerated, induced immunologic responses in a majority of patients, and results in disease regression in subset of patients. This brief report details the characterization of tumor cells and dendritic cells generated from patient BV01 with metastatic breast cancer following isolation from pleural effusions and leukapheresis, respectively.

Clinical Trial

Following completion of a review by the DOD, IRB and the FDA, patients were enrolled in the activated protocol and generations of DC/tumor fusion vaccine was initiated. During the last quarter, 5 patients have been enrolled in the trial. A detailed characterization of the tumor cells, the generated dendritic cells and the DC/tumor fusions pertaining to Patient BV01 was described in detail in the previous report. In the current report, we present detailed immune data following completion of vaccination of Patient BV01.

Immune monitoring of Patient BV01:

Induction of tumor-reactive lymphocytes by DC/breast cancer cell fusion vaccine

To assess the effects of fusion cell vaccination on the capacity of patients to mount a tumor-specific CTL response, we measured the immunologic response to vaccination by determining the percentage of circulating CD4⁺ and CD8⁺ T cells that recognize autologous breast carcinoma cells as manifested by the percentage of cells that express IFN- γ after ex-vivo exposure to autologous tumor lysate. Immunologic assessments were performed before each vaccination (three time points) and at one month after the last vaccine. The peak response after vaccination was compared with pre-vaccination levels of tumor-reactive T cells to assess the fold increase in tumor-reactive T cells after vaccination. At each time point, mononuclear cells were isolated from peripheral blood by Ficoll density centrifugation

and cryopreserved. After completion of the study, PBMCs were thawed, and 1×10^6 cells were cultured with lysate generated by repeated freeze/thaw cycles of 1×10^5 autologous breast carcinoma cells. As a control, PBMCs were cultured with tetanus toxoid ($10 \mu\text{g/mL}$) or media alone. After 5 days of coculture, expression of IFN- γ by CD4 $^+$ and CD8 $^+$ populations was determined by intracellular FACS analysis. Cells were restimulated with tumor lysate for 4 hours in the presence of $1 \mu\text{g/mL}$ GolgiStop (BD Biosciences). The cells were stained with FITC-conjugated CD4 or CD8 antibodies and permeabilized with Cytofix/Cytoperm Plus (BD Biosciences). Cells were thereafter stained with PE-conjugated anti-human IFN- γ , fixed in 2% paraformaldehyde, and analyzed by flow cytometry. A slight increase in the percentage of CD4 and CD8 T cells expressing IFN γ was observed at the pre-vaccination #2 time point, but this response tapered off after the patient received the third vaccine.

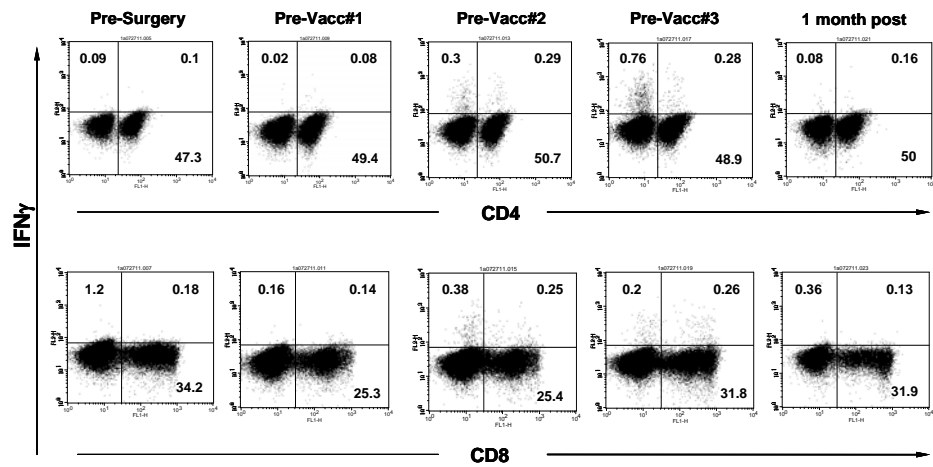


Figure 1. Expression of IFN γ by CD4 and CD8 populations before and after vaccination. PBMCs isolated before each vaccination and at serial time points after vaccination were cocultured with autologous tumor lysate, pulsed with GolgiStop, labeled with FITC-conjugated CD4 or CD8 antibodies, and then permeabilized by incubation in Cytofix/Cytoperm Plus. Cells were then incubated with PE conjugated anti-IFN γ or a matched isotype control antibody, fixed in 2% paraformaldehyde and thereafter analyzed by flow cytometry. Percentage of CD4 $^+$ (upper panel) and CD8 $^+$ T cells expressing IFN γ after ex vivo exposure to autologous tumor tumor lysate is shown. Numbers in the quadrants depict the percentage of cells positive.

Vaccine potency and Phytohemagglutinin and tetanus-induced induced T-cell proliferation

As a measure of potency of the generated vaccine as antigen-presenting cells, the capacity of the DC, breast carcinoma, and fusion cells to stimulate proliferation of allogeneic T cells was measured as shown below. In addition, freshly isolated patient PBMCs (1×10^5) isolated at serial time points (as outlined above) were cocultured in 200 μL of medium in 96-well U-bottomed plates for 4 days with 4 $\mu\text{g/mL}$ PHA and tetanus toxoid ($10 \mu\text{g/mL}$) (Figure 2, below). Proliferation was determined by measuring incorporation of [^3H]-Thymidine after overnight pulsing of triplicate samples. Data is presented as Stimulation Index. DC/Tumor fusion cells showed higher stimulation index as compared to tumor or dendritic cells alone. PHA stimulation was more enhanced at one month post-vaccination #3, whereas Tetanus Toxoid response to patient PMNCs did not change after the second vaccine as shown below.

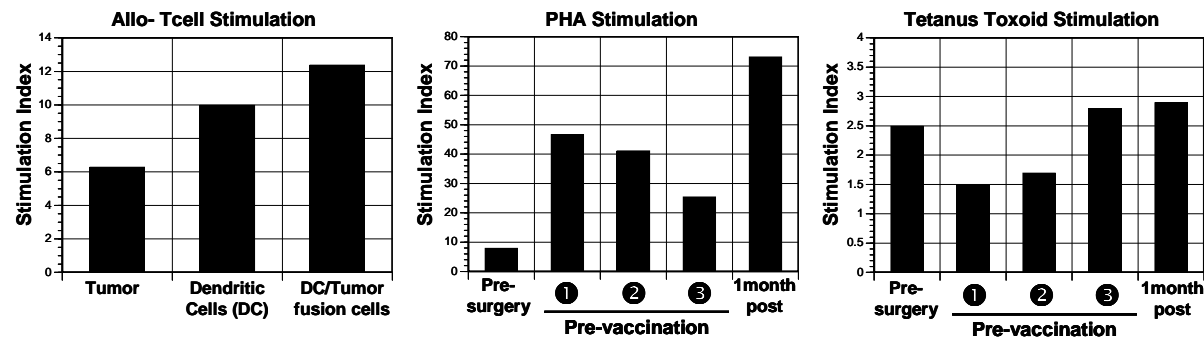


Figure 2. Potency of fusion cells in the stimulation of allogeneic T-cell proliferation and proliferation capacity of patient PBMCs pre- and post-vaccination. Patient-derived DCs, breast carcinoma cells, and fusion cells were cocultured with T cells from a healthy donor at a T-cell:target ratio of 1:10. Patient derived PBMCs at serial time points pre- and post-

vaccination were thawed and subjected to PHA and Tetanus Toxoid stimulation. Cocultures were incubated for 5 days, and T-cell proliferation was determined by incorporation of [³H]-thymidine (1 µCi/well) after overnight pulsing. Stimulation index (SI) represents counts per minute (CPM) of sample per CPM of unstimulated T cells.

Quantification of regulatory T cells

As outlined above, PBMCs were isolated at serial time points pre- and post-vaccination, and regulatory T cells were quantified by determining the percentage of CD4/CD25^{high} T cells by bidimensional FACS analysis (Figure 3). Expression of FOXP3 by CD4/CD25 cells was measured in by intracellular FACS analysis (Figure 4). No significant changes were observed in the expression of CD4/CD25^{high} T cells at pre-surgery and post-vaccination time points. Similarly, the expression of CD4/CD25 expressing FOXP3 did not change dramatically as depicted in Figure 3.

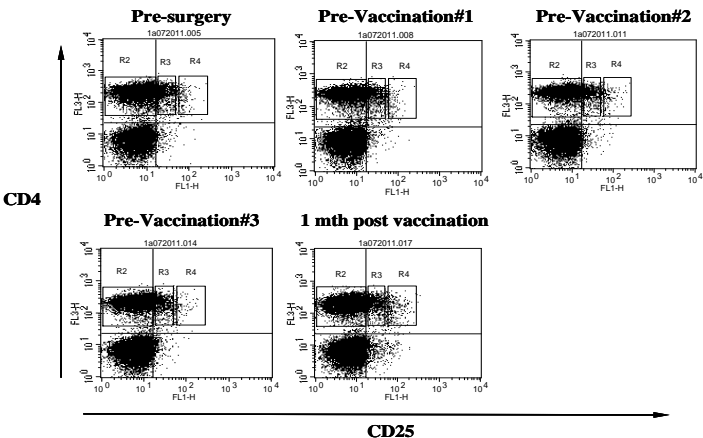


Figure 3. Phenotypic characterization of CD4⁺CD25⁻, CD4⁺CD25^{+low}, and CD4⁺CD25^{+high} T cells pre- and post-vaccination. Thawed PBMCs were cultured for 2 hours and then incubated with anti-CD4 TC and anti-CD25 FITC. Tcells were then separated into CD4⁺CD25⁻, CD4⁺CD25^{+low}, and CD4⁺CD25^{+high} fractions by bidimensional FACS sorting as shown in a representative dot plot. Quadrant gates were set up to distinguish the three observed populations of Tregs and the percentage of each cell type determined. The Table depicts the percentage of cells positive in each of the three quadrants at serial time points.

BV01-Tregs	Percentage of Cells positive		
	Gate R2	Gate R3	Gate R4
	CD4+Cd25-	CD4+CD25+low	CD4+CD25+high
Pre-Surgery	40.2	4.8	0.63
Pre-Vaccination # 1	39.4	4.9	0.63
Pre-Vaccination # 2	37.4	4.6	0.66
Pre-Vaccination # 3	35.8	3.8	0.38
1 mth post vaccination	39.1	3.5	0.86

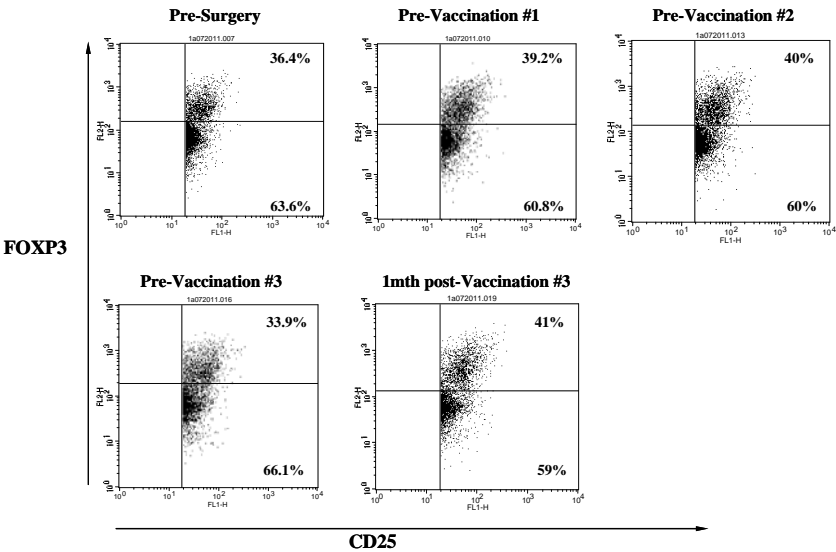


Figure 4. Thawed PBMCs at serial time points were cultured for 2 hours and stained with anti-CD4 TC and anti-CD25 FITC followed by treatment with Cytotfix Cytoperm Plus. Expression of FOXP3 by CD4/CD25 cells was measured by intracellular staining for FOXP3.

Conclusion

In the present study, patient BV01 with metastatic breast cancer was vaccinated with fusion cells generated from patient-derived tumor cells and autologous DCs. The results show that both CD4 and CD8 T cells expressed IFN γ post-vaccination suggesting an establishment of anti-tumor activity that tapered off at 1 month post-vaccination # 3. Furthermore, weak responses to PHA and Tetanus Toxoid were observed post vaccination, whereas, no dramatic change was observed in the levels of regulatory T cells or those that expressed FOXP3 pre- and post-vaccination.